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Maintaining Hepatic Stem Cell Gene Expression on Biological and Synthetic Substrata

Baltasar Lucendo-Villarin,¹ Ferdous Khan,² Salvatore Pernagallo,² Mark Bradley,² John P. Iredale,¹ and David C. Hay¹

Abstract

The liver is a highly resilient organ that possesses enormous regenerative capacity. This is mediated mainly through the most abundant cell type found in the liver, the hepatocyte. When the regenerative capacity of the hepatocyte is compromised, during chronic or acute liver injury, hepatic progenitor cells (HPCs) are activated to replace the damaged tissue. The HPC resides in a laminin-rich environment; as HPCs differentiate toward a hepatic or biliary fate, the extracellular matrix (ECM) composition changes, influencing cell behavior. To assess the impact that the biological ECM and the synthetic ECM have on the maintenance of hepatic stem cell gene expression, a murine hepatic stem cell line was employed. We demonstrate that hepatic stem cell gene expression could be maintained using a biological or synthetic substratum, but not on plastic alone.

Key words: biological; differentiation; liver; matrix; stem cell; synthetic

Introduction

THE LIVER HAS an exceptional regenerative capacity. After acute liver injury, hepatocytes undergo mitosis, leading to regeneration of the liver mass followed by functional restoration.¹ However, during chronic or severe acute liver injury, the regenerative capacity of resident hepatocytes is lost. In this situation, hepatic progenitor cells (HPCs) are activated to replace the damaged tissue. HPC populations are located at the most peripheral branches of the biliary tree, the Canals of Hering,² in a laminin-rich environment. The laminin-rich niche regulates the stem cell behavior, maintaining the balance between quiescence, proliferation, and differentiation required for tissue homeostasis and response to injury.³

HPCs first identified in rodents are termed oval cells (OCs) due to their ovoid shape.⁴ Previous studies have shown that OCs are bipotent and capable of differentiating toward either hepatocytes or cholangiocytes, reminiscent of hepatoblast differentiation during fetal liver development.^{5–7} OCs express adult hepatocyte markers such as albumin, cytokeratin 8 (CK8), CK18, alpha-1-antitrypsin, and hepatocyte nuclear factor 4 (HNF4). They also stain positively for biliary markers, including cytokeratins CK 7, CK 14, and CK 19, the OC marker OV-6, and alpha-fetoprotein (AFP).⁸ Additionally, OCs express adult hematopoietic cell markers such as *c-kit* and *Thy-1*.

OCs are a plastic cell population capable of self-renewal. These attributes make them an attractive cell population for use in cell-based therapy and the development of *in vitro* models. The development of predictive cell-based models is important in medicine, especially as OCs have been implicated in the formation of hepatocellular carcinoma. Therefore, a better understanding of OC malignant transformation, through *in vitro* modeling, may serve to identify more efficacious chemotherapeutic agents.^{9,10}

Essential to the development of cell-based therapies and predictive models is the robust delivery of stable cell populations that can be scaled up cost-effectively. The extracellular matrix (ECM) plays an essential part in this process. Therefore, the purpose of our study was to assess the suitability of a synthetic, inexpensive to manufacture, and totally defined surface for progenitor cell expansion.¹¹ To test this, we employed a bipotent murine stem cell line (bipotent mouse oval cell line [BMOL])¹² and compared the effects of different cellular substrata on stem cell gene expression.

Methods

Cell culture and seeding onto different matrices

BMOL was cultured on plastic (Corning) in the William's E medium (Gibco) supplemented with 2% fetal calf serum (Biosera) and 1% penicillin/streptomycin (Gibco). Media were

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TABLE 1. QIAGEN PRIMERS USED FOR QUANTITATIVE POLYMERASE CHAIN REACTION

Primer	Catalog number
<i>HNF4α</i>	QT00144739
<i>HNF1α</i>	QT00170975
<i>HNF1β</i>	QT00103320
<i>HNF6</i>	QT00297815
<i>SOX-9</i>	QT00163765
<i>PPIA</i>	QT00247709

HNF, hepatocyte nuclear factor; *PPIA*, peptidylprolyl isomerase A.

changed every second day. Cells were passaged using 0.05% trypsin (Gibco). For each experiment, 1×10^6 cells were plated onto BD cell culture plates coated with or without laminin (BD Biosciences). For experiments with the polymer, 5×10^5 cells were plated onto coverslips coated with synthetic polyurethane (PU134).¹¹ Cells were harvested by using trypsin for analysis 96 h postseeding.

RNA isolation and reverse transcription–polymerase chain reaction

About 1 μ g total RNA from the different BMOL cell populations was prepared using Qiagen Kit (Qiagen) and reverse-

transcribed following the manufacturer's instructions. Template cDNA, corresponding to 15 ng of RNA, was added to each polymerase chain reaction (PCR) and amplified using the QuantiFast SYBR assay (Qiagen) and QuantiTect (Qiagen). Genes used in this study are listed in Table 1. Each sample was run in triplicate for each candidate gene. Data were analyzed using LightCycler 480 Software (Roche), where expression levels of each gene of interest were normalized to peptidylprolyl isomerase A (*PPIA*). The two-tailed unpaired Student *t*-test was performed to test statistical significance, using Prism software (GraphPad Software).

Results

The effect of the cellular substrata on BMOL morphology and a stem cell marker expression

BMOLs were re-plated on different substrata: plastic, laminin, and PU134.¹¹ By phase-contrast microscopy, we observed that BMOLs maintained on either laminin or PU134 showed the typical cobblestone-like morphology, while maintenance on plastic induced the appearance of an elongated cellular morphology (Fig. 1A). These observations indicated that maintenance of the cells on different substrata was likely affecting stem cell gene expression. As a readout of the stem cell identity, we employed a well-established stem cell

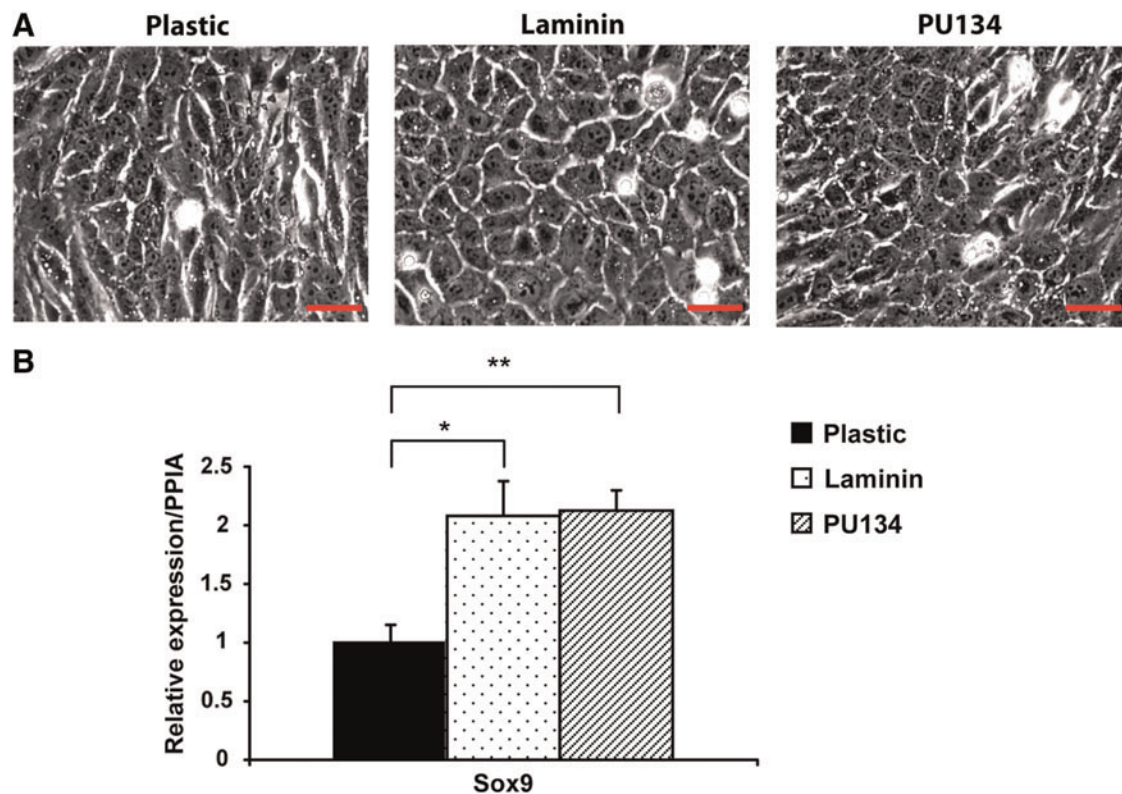


FIG. 1. The effect of the cellular substrata on the bipotent mouse oval cell line (BMOL) morphology and stem cell marker. **(A)** Phase-contrast microscopy images representative of the BMOL morphology on different matrices at $20\times$ magnification. The images were captured using an Axiocam MRC (Zeiss) connected to an Axiovert 200 inverted microscope (Zeiss). **(B)** Quantitative polymerase chain reaction of the stem cell marker *SOX-9* in BMOL cultured on different surfaces, showing a significant upregulation of *SOX-9* expression on cells maintained on laminin and PU134 in comparison with plastic. Relative expression refers to folds of induction of the gene compared with the endogenous gene control, peptidylprolyl isomerase A (*PPIA*). Data are expressed as mean \pm standard deviation (s.d.), * $p < 0.05$, ** $p < 0.01$, and $n = 3$. Scale bar represents 50 μ m.

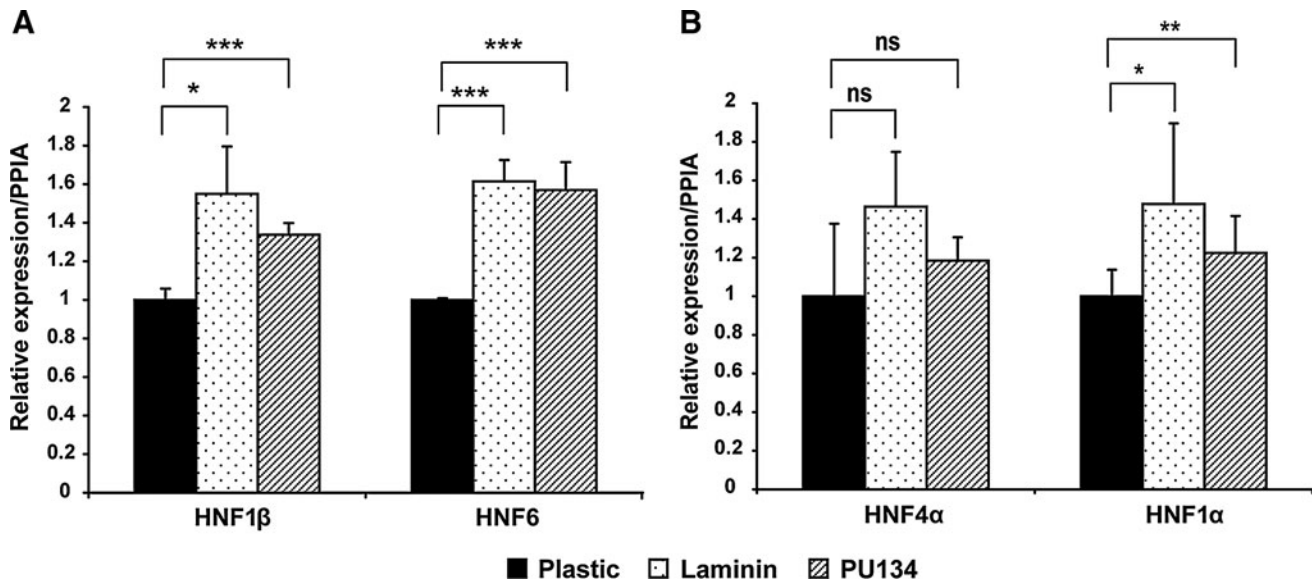


FIG. 2. The effect of the cellular substrata on BMOL bipotential gene expression. Quantitative polymerase chain reaction of biliary and hepatic marker expression in BMOL cultured on plastic, laminin, and PU134. **(A)** Expression of the biliary markers, hepatocyte nuclear factors *HNF1β* and *HNF6*, on cells maintained on different matrices. Both *HNF1β* and *HNF6* exhibited upregulation on laminin and PU134. **(B)** Expression of hepatic marker factors, *HNF4α* and *HNF1α*, on cells maintained on different matrices. *HNF1α* expression showed a significant upregulation on laminin and PU134. Nonsignificant (ns) change was observed for *HNF4α*. Relative expression refers to folds of induction of the gene compared with the endogenous gene control, PPIA. Data are expressed as mean \pm s.d., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and $n = 3$.

marker, *SOX-9*.^{13,14} *SOX-9* expression was assessed by quantitative PCR and increased significantly in cells maintained on PU134 (2.1-fold induction, $p < 0.01$) and laminin (1.8-fold induction, $p < 0.05$) when compared with cells maintained on plastic (Fig. 1B). These results also demonstrated that the PU134 substrate could support hepatic stem cell marker expression in a manner similar to laminin (Fig. 1B).

The effect of the cellular substrata on BMOL bipotential gene expression

Maintaining biliary and hepatic gene expression at appropriate levels is critical within bipotential progenitor cells. To study this relationship in more detail, BMOLs were re-plated on plastic, laminin, and PU134. It has been reported that the *HNF6* and *HNF1β* are important in biliary development,¹⁵ while *HNF1α* and *HNF4α* are important in hepatocyte specification.^{16,17} We assessed biliary and hepatic marker expression by quantitative PCR. We observed significant upregulation in *HNF6* expression on PU134 and laminin (both 1.6-fold induction, $p < 0.001$), and in *HNF1β* expression (1.4-fold, $p < 0.001$; and 1.6-fold, $p < 0.05$, respectively) when compared to the plastic controls (Fig. 2A). The hepatic marker *HNF1α* was also upregulated on laminin and PU134 (1.5-fold induction, $p < 0.05$; and 1.2-fold induction, $p < 0.01$, respectively). In contrast, *HNF4α* expression was similar between the different surfaces tested (Fig. 2B).

Discussion

This study explores the maintenance of bipotent stem cell gene expression on different ECMs. The ECM is known to play a crucial role in multiple biological processes, including liver progenitor cell self-renewal and differentiation.³ The results from our studies demonstrated that not only biological

matrices have profound effects on HPCs. We employed a synthetic matrix, PU134, which is known to promote hepatocyte differentiation and long-term stable drug-inducible function.¹¹ However, the ability of PU134 to support HPC gene expression had not been studied.

The bipotent murine oval cell line (BMOL) was employed throughout our studies. BMOLs were maintained on laminin, plastic, and PU134 surfaces. Four days post-replating, stem cell identity was determined by *SOX-9* expression. *SOX-9* gene expression was maintained on laminin and PU134, but not plastic (Fig. 1). In addition to stem cell marker expression, we also examined BMOL bipotential gene expression over the same time course. In contrast to plastic surfaces, BMOL cells maintained on either laminin or PU134 displayed maintenance of biliary and hepatic gene expression (Fig. 2).

The data presented demonstrates that both the laminin and PU134 supported BMOL stem cell and bipotential gene expression *in vitro*. These studies highlight the potential of synthetic matrices in cell biology and will likely improve cell culture definition, stability, scale-up, and reproducibility from a number of sources including: pluripotent stem cells and their derivatives; primary adult and fetal stem cells; and different somatic cell populations. This is essential if cell-based technologies are to be adopted by researchers and improve our understanding of the underlying biology in stem cell expansion, stem cell differentiation, human disease, human drug toxicity, and malignant transformation.

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Author Disclosure Statement

D.C.H. is CSO of, Director of, and a shareholder in FibromEd Ltd. J.P.I. and M.B. are shareholders in FibromEd Ltd.

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